NITROBLUE TETRAZOLIUM (NBT) - SUCCINATE VITAL STAINING OF AM FUNGI IN ROOTS

(some notes by C. Rivetta and R.M. Miller; comments on procedural details by L. Gades
Environmental Research Division, Argonne National Laboratory)

BACKGROUND

A procedure for the combination of vital staining and non-vital counterstaining of arbuscular mycorrhizal (AM) hyphae in fibrous roots is described. Vital staining with succinate-NBT reveals potential activity of AM fungal succinate dehydrogenase enzyme (SDH) in roots as purple drops of formazan (Mac Donald and Lewis, 1978; Kough et. al., 1987; Sylvia, 1988). Succinate dehydrogenase is a mitochondria enzyme that acts as a carrier for hydrogen removed in the aerobic oxidation of carbohydrate in the Krebs cycle. Pearse (1972) observed an increase in succinic oxidase activity for tissue in which the increased respiratory rate is part of general increase in activity. The presence of succinate dehydrogenase enzyme has been used to indicate the fungus metabolic activity (Kough et al., 1987; Hamel et al., 1990; Smith and Gianinazzi-Pearson, 1990; Smith and Dickson, 1991; Saito et al., 1993; Schaffer and Peterson, 1993; Tisserant et al., 1993; Smith et al., 1994). When root tissue is counterstained with non-vital acid fuchsin which stains the whole fungal structure pink by binding the acid fuchsin to cell wall material the SDH activity stands out particularly well.

The use of enzymes for accurate histochemical analysis requires control of all the variables capable of modifying the reaction, affecting the process of hydrolysis and subsequent staining. The most important factors are: enzyme concentration; time and temperature of incubation; pH at which the reaction is carried out; the presence of electrolytes, activators and inhibitors; and enzyme stability under the above conditions (Pearse, 1980).

The succinate dehydrogenase enzyme has the ability to remove hydrogen from one substrate (succinate) and transfer it to another (nitro blue tetrazolium salt). The dark coloration obtained in the fungus during the vital staining is due to the precipitation of reduced nitroblue tetrazolium salts as colored formazan in the form of purple drops.

Nitro-BT salts are colorless and act as electron acceptors in enzyme-catalyzed oxidation. Kuhn and Jerchel (1941) found that a number of colorless tetrazolium salts were reduced to colored compounds by plant tissues. The intact membrane of a mitochondrion constitutes a barrier to the penetration of the tetrazolium salt. The molecular size of all tetrazolium salts so far employed is sufficiently small for the diffusion constant to be high enough theoretically to allow rapid penetration into the tissues, but the penetration of Nitro-BT into the mitochondrion is

reduced to a low level and the amount of formazan is correspondingly small. This characteristic can be compensated for by incubating roots at high concentration of NBT (4 mg/ml).

It was also found that the effect of pH is not confined to altered kinetics of the enzyme reaction. There is also an effect on the mitochondria, at pH 7.4 and above very considerable swelling take place. When this occurs the velocity of oxidation of succinate is much faster (Pearse, 1972).

Another factor that affects the stability of the mitochondria is the presence or absence of Mg^{+2} (Pearse, 1968). The mechanism by which magnesium ions exert their protective effect is unexplained although it has been hypothesized that they bind ATP to the mitochondrion, thus preventing its loss. This protection is afforded by the use of five mM-magnesium ions in the incubating medium.

Nitro-BT is not lipid soluble, which is a disadvantage with regard to penetration of lipoprotein barriers, but it is offset by the fact that progressive formazans production in lipids droplets does not occur. The formazans from Nitro-BT appear to be insoluble in those lipids normally found in fungal tissues.

RECIPES FOR STOCK SOLUTIONS

Quantities are given for processing 100 samples.

- **Tris Buffer**: 6.56g Trizma acid, 0.97g Trizma base, add DI to a final volume (fv) of 250ml; adjust pH to 7.4.
- **NBT solution**: 1g NBT powder, add DI water to fv 250ml.
- **$MgCl_2$ solution**: 4.7605g $MgCl_2$ powder, add DI water to fv 100ml.
- **Formol Saline**: 100ml formaldehyde, 9.0g NaCl, 900ml DI water.
- **KOH**: 50g KOH crystals, add DI water to fv 1L.
- **Chloral hydrate solution**: 200g chloral hydrate crystals, add DI water to fv 1L
- **Ammonium peroxide solution**: 5ml NH_4OH, 16.7ml H_2O_2, DI water to fv 1L
- **Acid Fuchsin Solution**: 875ml lactic acid, 63ml glycerin, 63ml distilled water, 0.1g acid fuchsin

PREPARATION OF SECTIONS AND INCUBATION METHOD

- Collect fresh roots. If roots cannot be incubated immediately, keep them in the refrigerator in wet paper towels for up to a few hours. Wash and select about 0.25g (wet weight) of roots under 2mm diameter. Place in a capped 25ml centrifuge tube. [*Note: a recent study by Staddon and Fitter (2001) indicates that roots can be stored up to two weeks at 5°C without a decline in vitality.*]

- Incubate each sample for 24 hours in the dark at 37°C in:

2.5 ml Tris buffer (pH 7.4)
2.5 ml NBT solution
1.0 ml $MgCl_2$ solution
3.0 ml DI water
0.675 g Succinic acid

- Rinse 2-3 times in DI water.

Fixation

- Fix roots by placing each root sample in ~10ml formol saline solution in the dark at room temperature for at least 24 hours. After fixation, roots may be kept for several months in a refrigerator.

Clearing

- Rinse roots 2-3 times with DI water before clearing to remove formol saline.
- Transfer roots to heat-safe glass test tubes.
- Boil roots in ~10ml chloral hydrate for 10-15 min for grass roots or up to 1.5 hr for tree roots. Use a fume hood for this step. A dry bath or water bath may work well to maintain the temperature.
- Rinse 2-3 times in DI water.
- Incubate in ~10ml ammonium peroxide solution for 1 to 4.5 hours in the dark on a shaker (aluminum foil works well to block out light).
- Incubate in ~10ml KOH at 55°C for 24 hours for finer grass roots or up to 12 days for tree roots. Longer incubation times may require KOH to be changed every 2-3 days.

Acid Fuchsin Counterstaining

- Rinse roots 2-3 times in DI water
- Soak in 1% (v/v) HCl for 5 minutes
- Without rinsing, incubate in the dark at 55°C for one to two hours in acid fuchsin solution.

Storage of material

It is recommended that roots be mounted on slides and quantified shortly after acid fuchsin counterstaining. Roots may be stored short-term in glycerin. The fixation stage is the best time to store roots long-term until the investigator has time to counterstain, mount, and quantify.

MYCORRHIZAL QUANTIFICATION

- Arrange root segments lengthwise on the slide (we mounted 3 per slide).
- Add small drops of PVLG mounting solution.
- Slowly lower a cover slip from one end to the other, allowing PVLG to flow around the roots and air bubbles to escape. The flatter the roots, the easier quantification will be. The wooden end of a mounting needle can be used to this end; roll the wooden end gently over the cover slip or tap or push on the cover slip gently. A variety of methods can be used. This may be easier if PVLG is used sparingly or if slides have spent a few days drying.

The root length colonized by vesicular-arbuscular mycorrhizal fungi may be quantified with the magnified intersections method (160X and 250X) and expressed as a percentage of root length (McGongle et al., 1990). The field of view is moved across the slide, and a hairline graticule inserted into the eyepiece acts as the line of intersection with each root. Metabolically active structures appear as a dark blue-purple color; inactive structures appear bright pink. Each intersection may be quantified for:

Active hyphae	Non-active hyphae
Active coils	Non-active coils
Active arbuscules	Non-active arbuscules
Active vesicles	Non-active vesicles
Active arbuscules and hyphae	Non-active arbuscules and hyphae
Active vesicles and hyphae	Non-active vesicles and hyphae
Spores	Arbuscules and coils
*External hyphae only	External hyphae and other structures

*Note: external hyphae often stains as “inactive.”

A number of intersections (150 or more) is examined and recorded in the corresponding category. The total of intersections equals the sum of all categories. The percentage of root length colonized is calculated as follows:

Active colonization = $\sum$ (all active structures)/number of intersections counted

Total colonization = $\sum$ (all mycorrhizal structures)/number of intersections counted

Percentage root length colonized by each type of structure may be calculated in a similar fashion.

REFERENCES

Hamel C., Fyles H., Smith D.L. (1990) Measurement of development of endomycorrhizal mycelium using three different vital stains. New Phytol. 115: 297-302.

Kough J.L., Gianinazzi- Pearson V., and Gianinazzi S. (1987) Depressed metabolic activity of vesicular- arbuscular mycorrhizal fungi after fungicide applications. New Phytol. 106: 707-715.

MacDonald R.M. and Lewis M. (1978) The occurrences of some acid phosphatases and dehydrogenases in the vesicular-arbuscular mycorrhizal fungus *Glomus mosseae*. New Phytol. 80: 135-141.

McGonigle T.P., Miller M.H., Evans D.G., Fairchild G.L. and Swan. J.A. (1990) A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. New Phytol. 115: 495-501.

Pearse E.A.G. (1968) Histochemestry theoretical and applied, vol. 1, 3rd edn. Little, Brown & Company. Boston.

Pearse E.A.G. (1972) Histochemestry theoretical and applied, vol. 2, 3rd edn. The Williams & Wilkins Company. Baltimore.

Pearse E.A.G. (1980) Histochemestry theoretical and applied, vol. 1, 4th edn. Churchill Livingston. Edinburgh.

Saito M, Stribley D.P., and Hepper C.M. (1993) Succinate dehydrogenase activity of external and internal hyphae of a vesicular-arbuscular mycorrizal fungus, *Glomus mosseae* (Nicol. & Gerd) Gerdeman and Trappe, during mycorrhizal colonization of roots of leek (*Allium porrum* L.), as revealed by in situ histochemical staining. Mycorrhiza 4: 59-62.

Schaffer G.F. and Peterson R.L. (1993) Modifications to clearing methods used in combination with vital staining of roots colonized with vesicular-arbuscular mycorrhizal fungi. Mycorrhiza 4: 29-35.

Smith S.E. and Dickson S. (1991) Quantification of active vesicular-arbuscular mycorrhizal infection using image analysis and other techniques. Aust. J. Plant Physiol. 18: 637-648.

Smith S.E., Dickson S., Morris C. and Smith F.A. (1994) Transfer of phosphate from fungus to plant in VA mycorrhizas: calculation of the area of symbiotic interface and of the fluxes of P from two different fungi to *Allium porrum* L. New Phytol. 127: 93-99.

Smith S.E. and Gianinazzi-Pearson V. (1990) Phosphate uptake and arbuscular activity in mycorrhizal *Allium cepa* L.: effects of photon irradiance and phosphate nutrition. Aust. J. Plant Physiol. 17: 177-188.

Staddon P.L. and Fitter A.H. (2001) The differential vitality of intraradical mycorrhizal structures and its implications. Soil Biol. Biochem. 33: 129-132.

Sylvia D.M. (1988) Activity of external hyphae of vesicular-arbuscular mycorrhizal fungi. Soil Biol. Biochem. 20: 39-43.

Tisserant B., Gianinazzi-Pearson V., Gianinazzi S. and Gollotte A. (1993) *In planta* histochemical staining of fungal alkaline phosphatase activity for analysis of efficient arbuscular mycorrhizal infections. Mycol. Res. 97: 245-250.